

NUCLEIC ACID AND AMINO ACID SEQUENCES ENCODING  
HIGH-LEVEL EXPRESSOR FACTOR VIII POLYPEPTIDES  
AND METHODS OF USE

5 CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a U.S. Continuation of PCT Application No. PCT/US02/33403 filed on October 7, 2002, and which claims priority to U.S. Provisional Application No. 60/327,388, filed October 5, 2001, all of which are incorporated herein by reference in its entirety.

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FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

This work was supported by National Institute of Health Grant RO1 HL40921

FIELD OF THE INVENTION

15 The present invention relates the field of recombinant molecular biology, particularly a modified factor VIII polypeptide and methods of use.

BACKGROUND OF THE INVENTION

Factor VIII is a large (~ 300 kDa) glycoprotein that functions as an integral  
20 component of the intrinsic pathway of blood coagulation. It contains a series of domains designated A1-A2-B-*ap*-A3-C1-C2. The B domain of factor VIII has no known function and can be deleted without loss of coagulant activity. Mutations in the factor VIII gene that result in decreased or defective factor VIII protein give rise to the genetic disease, hemophilia A, which is characterized by recurrent bleeding  
25 episodes. Treatment of hemophilia A requires intravenous infusion of either plasma-derived or recombinant factor VIII.

Since the introduction of recombinant factor VIII for the treatment of hemophilia A, supply has struggled to keep up with demand because factor VIII is expressed and recovered at low levels in the heterologous mammalian cell culture  
30 systems used for commercial manufacture (Garber *et al.* (2000) *Nature Biotechnology* 18:1133). Additionally, factor VIII levels during hemophilia A gene therapy trials indicate that expression levels will be a limiting feature (Roth, *et al.* (2001) *N. Engl. J.*

Med. 344:1735-1742). The importance of this problem has resulted in significant research efforts to overcome the low factor VIII expression barrier. Several factors that limit expression have been identified, including low mRNA levels (Lynch *et al.* (1993) *Hum. Gene Ther.* 4:259-272; Hoeben *et al.* (1995) *Blood* 85:2447-2454; Koeberl *et al.* (1995) *Hum. Gene Ther.* 6:469-479), interaction with protein chaperones and inefficient secretion (Pipe *et al.* (1998) *J. Biol. Chem.* 273:8537-8544; Tagliavacca *et al.* (2000) *Biochemistry* 39:1973-1981; Kaufman *et al.* (1997) *Blood Coagul Fibrinolysis* 8 Suppl 2:S3-14) and rapid decay in the absence of von Willebrand factor (Kaufman *et al.* (1988) *J. Biol. Chem.* 263:6352-6362 and Kaufman *et al.* (1989) *Mol. Cell Biol.* 9:1233-1242). Deletion of the B-domain has been shown to increase factor VIII protein production in heterologous systems (Toole *et al.* (1986) *Proc. Natl. Acad. Sci. U.S.A.* 83:5939-5942). A B-domain deleted form of human factor VIII (Lind *et al.* (1995) *Eur. J. Biochem.* 232:19-27) has been approved for clinical use.

Despite these insights into factor VIII regulation, expression continues to be significantly lower than other recombinant proteins in the heterologous systems used in commercial manufacture (Kaufman *et al.* (1997) *Blood Coagul. Fibrinolysis* 8 Suppl 2:S3-14), as well as in *ex-vivo* (Roth, *et al.* (2001) *N. Engl. J. Med.* 344:1735-1742) and *in vivo* gene therapy applications (Chuah *et al.* (1995) *Hum. Gene Ther.* 6:1363-1377). Methods and compositions are needed for the increased expression of factor VIII.

## SUMMARY OF THE INVENTION

Methods and compositions are provided that allow for high-level expression of a factor VIII polypeptide. More specifically, the present invention provides methods and compositions comprising nucleic acid and amino acid sequences comprising a modified factor VIII that results in high-level expression of the polypeptide. The methods and compositions of the invention find use in the treatment of factor VIII deficiency, including, for example, hemophilia A.

In particular, one embodiment of the present invention provides an isolated polypeptide comprising an amino acid sequence set forth in SEQ ID NO:15, 17, or 19; an amino acid sequence having at least 85%, 90%, 95%, 97%, 98%, or 99% sequence

identity to SEQ ID NO:15, 17, or 19, wherein said polypeptide is characterized by high-level expression, or a fragment thereof.

In another embodiment of the invention, isolated nucleic acid molecules are provided comprising a nucleotide sequence set forth in SEQ ID NO:14, 16, or 18; a  
5 nucleotide sequence encoding a polypeptide comprising the amino acid sequence set forth in SEQ ID NO:15, 17, or 19; and, a nucleotide sequence having at least 85%, 90%, 95%, 97%, 98%, or 99% sequence identity to SEQ ID NO:14, 16, or 18, wherein said nucleotide sequence encodes a polypeptide that is characterized by high-level expression. Expression cassettes, vectors, and cells comprising the nucleic acid  
10 molecules of the invention are further provided.

Pharmaceutical compositions comprising the nucleic acid molecules and the polypeptides of the invention are also provided.

Methods for the production of a polypeptide are provided. In one embodiment, the method comprises introducing into a cell a nucleic acid molecule  
15 comprising a nucleotide sequence encoding a polypeptide comprising the amino acid sequence set forth in SEQ ID NO:15, 17, or 19; a nucleotide sequence comprising the sequence set forth in SEQ ID NO:14, 16, or 18; a nucleotide sequence having at least 85%, 90%, 95%, 97%, 98%, or 99% sequence identity to SEQ ID NO:14, 16, or 18, wherein the nucleotide sequence encodes a polypeptide characterized by high-level  
20 expression, or a fragment thereof; and, culturing the cell under conditions that allow expression of the nucleotide sequence.

Also provided are methods for increasing the level of expression of the factor VIII polypeptide. In one embodiment, the method comprises introducing into a cell a nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide  
25 comprising the amino acid sequence set forth in SEQ ID NO:15, 17, or 19; a nucleotide sequence comprising the sequence set forth in SEQ ID NO:14, 16, or 18; a nucleotide sequence having at least 85%, 90%, 95%, 97%, 98%, or 99% sequence identity to SEQ ID NO:14, 16, or 18, wherein the nucleotide sequence encodes a polypeptide characterized by high-level expression, or a fragment thereof; and,  
30 culturing the cell under conditions that allow expression of the nucleotide sequence.

Also provided is a method for the treatment of factor VIII deficiencies, including, for example, hemophilia A. The method comprises administering to a subject in need thereof a composition comprising a therapeutically effective amount

of a polypeptide, where the polypeptide comprises an amino acid sequence set forth in SEQ ID NO:15, 17, or 19, an amino acid sequence having at least 85%, 90%, 95%, 97%, 98%, or 99% sequence identity to SEQ ID NO:15, 17, or 19, wherein said polypeptide is characterized by high-level expression, or a fragment thereof.

5           Other methods include treating a factor VIII deficiency by administering to a subject in need thereof a composition comprising a therapeutically effective amount of a nucleic acid molecule, where said nucleic acid molecule comprises a nucleotide sequence set forth in SEQ ID NO:14, 16, or 18; a nucleotide sequence encoding a polypeptide comprising the amino acid sequence set forth in SEQ ID NO:15, 17, or  
10   19; a nucleotide sequence having at least 85%, 90%, 95%, 97%, 98%, or 99% sequence identity to SEQ ID NO:14, 16, or 18, wherein said nucleic acid molecule encodes a polypeptide characterized by high-level expression, or a fragment thereof.

#### BRIEF DESCRIPTION OF THE DRAWINGS

15           Figure 1A-1H taken together provide an aligned amino acid sequence comparison of the human (SEQ ID NO:6), porcine (SEQ ID NO:2), and mouse (SEQ ID NO:8) factor VIII polypeptide sequences.

            Figure 2 provides a schematic of B domain-deleted human, porcine, and hybrid human/porcine factor VIII constructs. The solid line between the A2 and *ap*  
20   domains represents linker sequences.

            Figure 3 provides graphical data showing heterologous expression of a recombinant B domain-deleted porcine factor VIII protein, designated P/OL, recombinant B domain-deleted human factor VIII protein, and five recombinant B domain-deleted hybrid human/porcine factor VIII proteins, designated HP1, HP30,  
25   HP44, HP46, and HP47. COS-7 cells (solid bars) and baby hamster kidney-derived cells, designated BMK-M cells, (hatched bars) were transfected with the individual factor VIII expression constructs and luciferase plasmid DNA and cultured in serum-free media for 24 hr. The data illustrates that there is a significant increase in expression of P/OL, HP44, HP47, and HP46 compared to HSQ. In contrast,  
30   expression of HP1 and HP30 were not increased compared to HSQ.

            Figure 4 provides the amino acid sequence for the factor VIII<sub>SEP</sub> polypeptide designated herein as HP44/OL (SEQ ID NO:15).

Figure 5A-5D provides the nucleotide sequence (SEQ ID NO: 14) encoding the factor VIII<sub>SEP</sub> polypeptide designated herein as HP44/OL.

Figure 6 provides the amino acid sequence for the factor VIII<sub>SEP</sub> polypeptide designated herein as HP46/SQ (SEQ ID NO:17).

5        Figure 7A-7D provides the nucleotide sequence (SEQ ID NO: 16) encoding the factor VIII<sub>SEP</sub> polypeptide designated herein as HP46/SQ.

Figure 8 provides the amino acid sequence for the factor VIII<sub>SEP</sub> polypeptide designated herein as HP47/SQ (SEQ ID NO:19).

10       Figure 9A-9D provides the nucleotide sequence (SEQ ID NO: 18) encoding the factor VIII<sub>SEP</sub> polypeptide designated herein as HP47/SQ.

Figure 10A-10D provides the amino acid sequence for the human factor VIII B-domain deleted polypeptide (SEQ ID NO:13).

Figure 11A-11B provides the nucleotide sequence (SEQ ID NO:12) encoding the human factor VIII B-domain deleted polypeptide.

15       Figure 12 provides a schematic representation of one possible factor VIII<sub>SEP</sub> variant of the present invention. The variant, referred to as HP63/OL, contains the porcine A1 domain and a partially humanized *ap*-A3 domain that comprises porcine amino acids from about 1690 to about 1804 and from about 1819 to about 2019.

20       Figure 13 provides the amino acid sequence (SEQ ID NO:21) encoding the factor VIII<sub>SEP</sub> polypeptide designated herein as HP63/OL.

Figure 14A-14B provides the nucleotide sequence (SEQ ID NO: 20) encoding the factor VIII<sub>SEP</sub> polypeptide designated herein as HP63/OL.

## DETAILED DESCRIPTION OF THE INVENTION

### 25    Overview

The present invention provides methods and compositions that allow for high-level expression of the factor VIII polypeptide. The factor VIII polypeptide contains homology-defined proteins domains having the following nomenclature: A1-A2-B-*ap*-A3-C1-C2. The present invention has identified regions within the domains of a non-human factor VIII polypeptide that promote high-level expression of the factor VIII polypeptide. More particularly, regions of the porcine factor VIII polypeptide that comprises the A1 and *ap*-A3 regions, and variants and fragments thereof, have been identified which impart high-level expression to both the porcine and human

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factor VIII polypeptide. The present invention thus provides methods and compositions that use the non-human factor VIII polypeptide sequences which impart high-level expression, and active variants or fragments of these sequences, to construct nucleic acid and polypeptide sequences encoding a modified factor VIII polypeptide that results in high-level expression of the encoded factor VIII polypeptide. The modified factor VIII polypeptides characterized by high-level expression are referred to herein as "factor VIII<sub>SEP</sub>" (Super Expression).

By "high-level expression" is intended the production of a polypeptide at increased levels when compared to the expression levels of the corresponding human factor VIII polypeptide expressed under the same conditions. An increase in polypeptide levels (i.e., high-level expression) comprises at least about 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 20 fold or greater expression of the factor VIII<sub>SEP</sub> polypeptide compared to the expression levels of the corresponding human factor VIII polypeptide. Alternatively, "high-level expression" can comprise an increase in polypeptide expression levels of at least 1-25 fold, 1-5 fold, 5-10 fold, 10-15 fold, 15-20 fold, 20-25 fold or greater expression levels of the factor VIII<sub>SEP</sub> when compared to the corresponding human factor VIII polypeptide. Methods for assaying "high-level expression" are routine in the art and are outlined in more detail below.

By "corresponding" factor VIII polypeptide is intended a factor VIII polypeptide that comprises an equivalent amino acid sequence. For instance, when a modified factor VIII polypeptide comprising the A1-A2-*ap*-A3-C1-C2 domains is tested for high-level expression, a human or porcine factor VIII polypeptide containing corresponding domains will be used (i.e., A1-A2-*ap*-A3-C1-C2). When a fragment of a modified factor VIII polypeptide is tested for high-level expression (i.e., A1-A2-*ap*-A3), a human or porcine factor VIII polypeptide having the corresponding domains will be tested (i.e., A1-A2-*ap*-A3).

### Compositions

Compositions of the invention include the nucleic acid molecules encoding factor VIII polypeptides characterized by high-level expression. As outlined in further detail below, the A1 domain of porcine factor VIII (amino acid residues 20-391 of SEQ ID NO:19) and the *ap*-A3 domain of porcine factor VIII (amino acids 1450-1820 of SEQ ID NO:19) allow for high-level expression of factor VIII. The

present invention thus provides methods and compositions comprising factor VIII<sub>SEP</sub> polypeptides and active variant and active fragments of factor VIII<sub>SEP</sub> polypeptides characterized by high-level expression.

In particular, the present invention provides for isolated nucleic acid molecules comprising nucleotide sequences encoding the amino acid sequence shown in SEQ ID NOS:15, 17, or 19 and active fragments or active variants thereof. Also provided are isolated nucleic acid molecules comprising nucleotide sequences set forth in SEQ ID NOS:14, 16, or 18 and active fragments or active variants thereof. Further provided are polypeptides having an amino acid sequence encoded by a nucleic acid molecule described herein, for example, those set forth in SEQ ID NOS:15, 17, and 19 and active fragments and active variants thereof.

Table 1 provides a summary of structure of the sequences provided in SEQ ID NOS:14-19, where the subscript "P" designates a domain from porcine factor VIII and the subscript "H" designates a domain from human factor VIII.

Table 1. Summary of Sequence Structure

| SEQ ID NO | Factor VIII domains  |
|-----------|--|
| 14 and 15 | A1 <sub>P</sub> -A2 <sub>P</sub> -ap <sub>P</sub> -A3 <sub>P</sub> -C1 <sub>H</sub> -C2 <sub>H</sub> |
| 16 and 17 | A1 <sub>P</sub> -A2 <sub>H</sub> -ap <sub>H</sub> -A3 <sub>H</sub> -C1 <sub>H</sub> -C2 <sub>H</sub> |
| 18 and 19 | A1 <sub>P</sub> -A2 <sub>H</sub> -ap <sub>P</sub> -A3 <sub>P</sub> -C1 <sub>H</sub> -C2 <sub>H</sub> |

The invention encompasses isolated or substantially purified nucleic acid or protein compositions. An "isolated" or "purified" nucleic acid molecule or protein, or biologically active portion thereof, is substantially free of other cellular material, or culture medium when produced by recombinant techniques, or substantially free of chemical precursors or other chemicals when chemically synthesized. Preferably, an "isolated" nucleic acid is free of sequences (preferably protein encoding sequences) that naturally flank the nucleic acid (i.e., sequences located at the 5' and 3' ends of the nucleic acid) in the genomic DNA of the organism from which the nucleic acid is derived. For example, in various embodiments, the isolated nucleic acid molecule can contain less than about 5 kb, 4 kb, 3 kb, 2 kb, 1 kb, 0.5 kb, or 0.1 kb of nucleotide sequences that naturally flank the nucleic acid molecule in genomic DNA of the cell from which the nucleic acid is derived. A protein that is substantially free of cellular material includes preparations of protein having less than about 30%, 20%, 10%, 5%, (by dry weight) of contaminating protein. When the protein of the invention or

RTA01/2152497v1

biologically active portion thereof is recombinantly produced, preferably culture medium represents less than about 30%, 20%, 10%, or 5% (by dry weight) of chemical precursors or non-protein-of-interest chemicals.

5 Fragments and variants of the disclosed factor VIII<sub>SEP</sub> nucleotide sequences and proteins encoded thereby are also encompassed by the present invention. By “fragment” is intended a portion of the nucleotide sequence or a portion of the amino acid sequence and hence protein encoded thereby. Fragments of a nucleotide sequence may encode protein fragments that retain the biological activity of the polypeptides set forth in SEQ ID NO:15, 17, or 19 and hence are characterized by  
10 high-level expression of the factor VIII polypeptide. Thus, fragments of a nucleotide sequence may range from at least about 10, about 20 nucleotides, about 50 nucleotides, about 100 nucleotides, about 500 nucleotides, about 1000 nucleotides, about 2000 nucleotides, about 3000 nucleotides, about 4000 nucleotides, about 5000 nucleotides, about 6000 nucleotides, about 7000 nucleotides, about 8000 nucleotides,  
15 and up to the full-length nucleotide sequence encoding the factor VIII polypeptide of the invention about 9000 nucleotides.

A fragment of a nucleotide sequence of the present invention that encodes a biologically active portion of a factor VIII<sub>SEP</sub> protein of the invention will encode at least 12, 25, 30, 50, 100, 150, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1100,  
20 1200, 1300, 1400, 1500, 1600, 1700, 1800, 1900, 2000, 2100, 2200, 2300 contiguous amino acids, or up to the total number of amino acids present in a full-length factor VIII protein of the invention (for example, 1457, 1467, or 1467 amino acids for SEQ ID NO:15, 17, or 19 respectively) and will allow high-level expression of the factor VIII polypeptide.

25 By “variant” is intended substantially similar sequences. For nucleotide sequences, conservative variants include those sequences that, because of the degeneracy of the genetic code, encode the amino acid sequence of one of the polypeptides of the invention. Variant nucleotide sequences include synthetically derived nucleotide sequences, such as those generated, for example, by using site-  
30 directed mutagenesis but which still encode a factor VIII<sub>SEP</sub> protein characterized by high-level expression. Generally, variants of a particular nucleotide sequence of the invention will have at least at least 75%, 80%, 85%, 86%, 87%, 88%, 89%, preferably about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, and more preferably about 98%,



99%, or more sequence identity to that particular nucleotide sequence as determined by sequence alignment programs described elsewhere herein.

By “variant” protein is intended a protein derived from the polypeptide of SEQ ID NO:15, 17, or 19 by deletion (so-called truncation) or addition of one or more amino acids to the N-terminal and/or C-terminal end of the protein; deletion or addition of one or more amino acids at one or more sites in the protein; or substitution of one or more amino acids at one or more sites in the protein. Variant proteins encompassed by the present invention are biologically active, that is they continue to possess the desired biological activity of SEQ ID NO:15, 17, or 19, hence they will continue to allow for the high-level expression of the factor VIII polypeptide. Such variants may result from, for example, genetic polymorphism or from human manipulation. Biologically active variants of a polypeptide of the invention will have at least 75%, 80%, 85%, 86%, 87%, 88%, 89%, preferably about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to the amino acid sequence for SEQ ID NO:15, 17, or 19 as determined by sequence alignment programs described elsewhere herein using default parameters. A biologically active variant of a protein of the invention may differ from that protein by as few as 1-100, 1-50, 1-25, 1-15 amino acid residues, as few as 1-10, such as 6-10, as few as 5, as few as 4, 3, 2, or even 1 amino acid residue.

Biological activity of the factor VIII<sub>SEP</sub> polypeptides of the present invention can be assayed by any method known in the art. As discussed above, the factor VIII<sub>SEP</sub> polypeptides of the invention are characterized by high-level expression. Assays to measure high-level expression are known in the art. For example, the level of expression of the factor VIII<sub>SEP</sub> polypeptide can be measured by Western blot analysis or ELISA. Other methods include, for example, labeling cell lines expressing the factor VIII polypeptide with <sup>35</sup>S-methionine, followed by immunoprecipitation of radiolabeled factor VIII molecules. Alternatively, the level of expression of the factor VIII<sub>SEP</sub> polypeptide can be assayed for by measuring the activity of the factor VIII polypeptide. For example, increased factor VIII expression could be assayed by measuring factor VIII activity using standard assays known in the art, including a one-stage coagulation assay or a two-stage activity assay. See, for example, U.S. Patent No. 6,458,561 and the Experimental section below.

Briefly, coagulation assays are based on the ability of factor VIII to shorten the clotting time of plasma derived from a patient with hemophilia A. For example, in the one-stage assay, 0.1 ml hemophilia A plasma (George King Biomedical, Inc.) is incubated with 0.1 ml activated partial thromboplastin reagent (APTT) (Organon  
5 Teknika) and 0.01 ml sample or standard, consisting of diluted, citrated normal human plasma, for 5 min at 37° C in a water bath. Incubation is followed by addition of 0.1 ml 20 mM CaCl<sub>2</sub>, and the time for development of a fibrin clot is determined by visual inspection. A unit of factor VIII is defined as the amount present in 1 ml of citrated normal human plasma.

10       The one-stage assay relies on endogenous activation of factor VIII by activators formed in the hemophilia A plasma, whereas the two-stage assay measures the procoagulant activity of preactivated factor VIII. In the two-stage assay, samples containing factor VIII that are reacted with thrombin are added to a mixture of activated partial thromboplastin and human hemophilia A plasma that is preincubated  
15 for 5 min at 37° C. The resulting clotting times are converted to units/ml, based on the same human standard curve described above. See, for example, U.S. Patent No. 6,376,463.

      The factor VIII<sub>SEP</sub> polypeptides of the invention may be altered in various ways including amino acid substitutions, deletions, truncations, and insertions.  
20       Methods for such manipulations are generally known in the art. For example, amino acid sequence variants of the factor VIII<sub>SEP</sub> proteins can be prepared by mutations in the DNA. Methods for mutagenesis and nucleotide sequence alterations are well known in the art. See, for example, Kunkel (1985) *Proc. Natl. Acad. Sci. USA* 82:488-492; Kunkel *et al.* (1987) *Methods in Enzymol.* 154:367-382; US Patent No.  
25 4,873,192; Walker and Gaastra, eds. (1983) *Techniques in Molecular Biology* (MacMillan Publishing Company, New York) and the references cited therein. Guidance as to appropriate amino acid substitutions that do not affect biological activity (i.e., high-level expression) of the factor VIII<sub>SEP</sub> may be found in the model of Dayhoff *et al.* (1978) *Atlas of Protein Sequence and Structure* (Natl. Biomed. Res.  
30 Found., Washington, D.C.), herein incorporated by reference. Conservative substitutions, such as exchanging one amino acid with another having similar properties, may be preferred. Alternatively, methods to minimize the number of porcine amino acids in the A<sub>1</sub> and *ap*-A<sub>3</sub> domains of factor VIII<sub>SEP</sub> and still continue

to retain the high-level expression of the factor VIII<sub>SEP</sub> are known in the art and include, for example, established site-directed mutagenesis such as by splicing overlap extension as described elsewhere herein. Obviously, the mutations that will be made in the DNA encoding the variant must not place the sequence out of reading  
5 frame and preferably will not create complementary regions that could produce secondary mRNA structure. See, EP Patent Application Publication No. 75,444.

When it is difficult to predict the exact effect of the substitution, deletion, or insertion in advance of doing so, one skilled in the art will appreciate that the effect will be evaluated by routine screening assays. That is, the activity can be evaluated  
10 by high-level expression of the factor VIII polypeptide as discussed in detail elsewhere herein.

By "sequence identity" is intended the same nucleotides or amino acid residues are found within the variant sequence and a reference sequence when a specified, contiguous segment of the nucleotide sequence or amino acid sequence of  
15 the variant is aligned and compared to the nucleotide sequence or amino acid sequence of the reference sequence. Methods for sequence alignment and for determining identity between sequences are well known in the art. See, for example, Ausubel *et al.*, eds. (1995) *Current Protocols in Molecular Biology, Chapter 19* (Greene Publishing and Wiley-Interscience, New York); and the ALIGN program  
20 (Dayhoff (1978) in *Atlas of Polypeptide Sequence and Structure 5:Suppl. 3* (National Biomedical Research Foundation, Washington, D.C.). With respect to optimal alignment of two nucleotide sequences, the contiguous segment of the variant nucleotide sequence may have additional nucleotides or deleted nucleotides with respect to the reference nucleotide sequence. Likewise, for purposes of optimal  
25 alignment of two amino acid sequences, the contiguous segment of the variant amino acid sequence may have additional amino acid residues or deleted amino acid residues with respect to the reference amino acid sequence. The contiguous segment used for comparison to the reference nucleotide sequence or reference amino acid sequence will comprise at least 20 contiguous nucleotides, or amino acid residues, and may be  
30 30, 40, 50, 100, or more nucleotides or amino acid residues. Corrections for increased sequence identity associated with inclusion of gaps in the variant's nucleotide sequence or amino acid sequence can be made by assigning gap penalties. Methods of sequence alignment are well known in the art.

The determination of percent identity between two sequences is accomplished using a mathematical algorithm. Specifically, for the purpose of the present invention percent identity of an amino acid sequence is determined using the Smith-Waterman homology search algorithm using an affine 6 gap search with a gap open penalty of 12  
5 and a gap extension penalty of 2, BLOSUM matrix 62. The Smith-Waterman homology search algorithm is taught in Smith and Waterman (1981) *Adv. Appl. Math* 2:482-489, herein incorporated by reference. Alternatively, for the purposes of the present invention percent identity of a nucleotide sequence is determined using the Smith-Waterman homology search algorithm using a gap open penalty of 25 and a  
10 gap extension penalty of 5. Such a determination of sequence identity can be performed using, for example, the DeCypher Hardware Accelerator from TimeLogic.

It is further recognized that when considering percentage of amino acid identity, some amino acid positions may differ as a result of conservative amino acid substitutions, which do not effect the properties of polynucleotide function. In these  
15 instances, percent sequence identity may be adjusted upwards to account for the similarity in conservatively substituted amino acids. Such adjustments are well known in the art. See, for example, Meyers *et al.* (1988) *Computer Applic. Biol. Sci.* 4:11-17.

It is recognized that variants of sequences of the invention may encode factor  
20 VIII<sub>SEP</sub> polypeptides that contain only the amino acid residues of the A1<sub>P</sub> and ap-A3<sub>P</sub> domains that confer the high-level expression to the factor VIII polypeptide. Consequently, the A1<sub>P</sub> and A3<sub>P</sub> domains can be progressively humanized such that only the residues required to retain high-level expression are retained in the factor VIII<sub>SEP</sub> polypeptide. Such methods are known by those skilled in the art and also  
25 discussed in more detail below. See also, for example, U.S. Patent Nos. 6,376,463; 6,48,563; 5,744,466; 5,888,974; 5,663,060; 5,364,771; 5,859,204; and, 6,180,371; all of which are herein incorporated by reference. In addition, it is recognized that a three-dimensional model of the human factor VIII A1-A2-A3 domains can be used to identify regions away from the domain interface. One of skill will be able to use this  
30 model to identify target amino acids residues for humanization.

It is recognized that the variant factor VIII<sub>SEP</sub> or fragments thereof can be made (1) by substitution of isolated, plasma-derived animal subunits or human subunits (heavy or light chains) for corresponding human subunits or animal subunits;

(2) by substitution of human domains or animal domains (A1, A2, A3, B, C1, and C2) for corresponding animal domains or human domains; (3) by substitution of parts of human domains or animal domains for parts of animal domains or human domains; (4) by substitution of at least one specific sequence including one or more unique human or animal amino acid(s) for the corresponding animal or human amino acid(s); or (5) by substitution of amino acid sequence that has no known sequence identity to factor VIII for at least one sequence including one or more specific amino acid residue(s) in human, animal, or variant factor VIII or fragments thereof. Individual amino acid replacements can be obtained by site-directed mutagenesis of the corresponding segment coding DNA.

In a factor VIII molecule, a "domain", as used herein, is a continuous sequence of amino acids that is defined by internal amino acid sequence identity and sites of proteolytic cleavage by thrombin. Unless otherwise specified, factor VIII domains include the following amino acid residues, when the sequences are aligned with the human amino acid sequence (SEQ ID NO:6): A1, residues Ala1-Arg372; A2, residues Ser373-Arg740; B, residues Ser741-Arg1648; A3, residues Ser1690-Ile2032; C1, residues Arg2033-Asn2172; C2, residues Ser2173-Tyr2332. The A3-C1-C2 sequence includes residues Ser1690-Tyr2332. The remaining sequence, residues Glu1649-Arg1689, is usually referred to as the factor VIII light chain activation peptide. Factor VIII is proteolytically activated by thrombin or factor Xa, which dissociates it from von Willebrand factor, forming factor VIIIa, which has procoagulant function. The biological function of factor VIIIa is to increase the catalytic efficiency of factor IXa toward factor X activation by several orders of magnitude. Thrombin-activated factor VIIIa is a 160 kDa A1/A2/A3-C1-C2 heterotrimer that forms a complex with factor IXa and factor X on the surface of platelets or monocytes. A "partial domain" as used herein is a continuous sequence of amino acids forming part of a domain. "Subunits" of human or animal (i.e., mouse, pig, dog etc.) factor VIII, as used herein, are the heavy and light chains of the protein. The heavy chain of factor VIII contains three domains, A1, A2, and B. The light chain of factor VIII also contains three domains, A3, C1, and C2. A "unique" amino acid residue or sequence, as used herein, refers to an amino acid sequence or residue in the factor VIII molecule of one species that is different from the homologous residue or sequence in the factor VIII molecule of another species. As used herein, "mammalian factor VIII" includes factor VIII with

amino acid sequence derived from any non-human mammal, unless otherwise specified. "Animal", as used herein, refers to pig and other non-human mammals.

Since current information indicates that the B domain has no inhibitory epitope and has no known effect on factor VIII function, factor VIII<sub>SEP</sub> variants of the present invention may have a B domain or a portion thereof. In addition, factor VIII<sub>SEP</sub> variants may also have the factor VIII B-domain with the B-domain from porcine or human factor V. See, for example, U.S. Patent No. 5,004,803. A "B-domainless" variant factor VIII<sub>SEP</sub> or fragment thereof, as used herein, refers to any one of the variant factor VIII<sub>SEP</sub> constructs described herein that lacks the B domain, or a portion thereof.

One of skill in the art will be aware of techniques that allow individual subunits, domains, or continuous parts of domains of animal or human factor VIII cDNA to be cloned and substituted for the corresponding human or porcine subunits, domains, or parts of domains by established mutagenesis techniques and thereby generate a factor VIII<sub>SEP</sub> or variant or fragment thereof. For example, Lubin *et al.* (1994) *J. Biol. Chem.* 269(12):8639-8641 describes techniques for substituting the porcine A2 domain for the human domain using convenient restriction sites. Other methods for substituting a region of the factor VIII cDNA of one species for the factor VIII cDNA of another species include splicing by overlap extension ("SOE"), as described by Horton *et al.* (1993) *Meth. Enzymol* 217:270-279.

#### DNA constructs and Vectors

The nucleotide sequence encoding the factor VIII<sub>SEP</sub> polypeptides or active variants or fragments thereof can be contained in a DNA construct. The DNA construct can include a variety of enhancers/promoters from both viral and mammalian sources that drive expression of the factor VIII<sub>SEP</sub> polypeptide in the desired cell type. The DNA construct can further contain 3' regulatory sequences and nucleic acid sequences that facilitate subcloning and recovery of the DNA.

The transcriptional promoter and, if desired, the transcriptional enhancer element are operably linked to the nucleic acid sequence of the factor VIII polypeptide. A "promoter" is defined as a minimal DNA sequence that is sufficient to direct transcription of a nucleic acid sequence. A "transcriptional enhancer element" refers to a regulatory DNA sequence that stimulates the transcription of the adjacent

gene. The nucleic acid sequence encoding the factor VIII polypeptide is operably linked to the promoter sequence. See, for example, Goeddel (1990) *Gene Expression Technology: Methods in Enzymology 185* (Academic Press, San Diego, CA).

By “operably linked” is intended a functional linkage between the regulatory promoter and the nucleic acid sequence encoding the factor VIII polypeptide. The functional linkage permits gene expression of factor VIII when the appropriate transcription activator proteins are present.

Thus, the DNA construct can include a promoter that may be native or foreign. By “foreign” it is meant a sequence not found in the native organism. Furthermore, the transcription regulatory elements may be heterologous to the nucleotide sequence encoding factor VIII. By “heterologous” is intended any nucleotide sequence not naturally found upstream of the sequence encoding the factor VIII polypeptide. The promoter may be a natural sequence or a synthetic sequence. In addition, the promoter may be constitutively active, tissue-specific, or inducible. A tissue-specific promoter is preferentially activated in a given tissue and results in expression of a gene product in the tissue where activated.

For use in mammalian cells, the promoters may be derived from a virus. For example, commonly used promoters are derived from polyoma, Simian Virus 40 (SV40) and Adenovirus 2. The early and late promoters of SV40 virus are useful as is the major late promoter of adenovirus. Further, it is also possible, and often desirable, to utilize promoter or control sequences normally associated with the desired gene sequence, provided such control sequences are compatible with the host cell system.

In certain embodiments, the introduction of the nucleotide sequence encoding factor VIII into a cell can be identified *in vitro* or *in vivo* by including a marker in the DNA construct. The marker will result in an identifiable change in the genetically transformed cell. Drug selection markers include for example neomycin, puromycin, hygromycin, DHFR, GPT, zeocin and histidinol. Alternatively, enzymes such as herpes simplex virus thymidine kinase (TK) or immunological markers can be used. Further examples of selectable markers are well known in the art.

It is recognized that multiple alterations can be envisioned for the design of the DNA construct used in the methods of the present invention. For instance, the construct may be designed for the insertion of the nucleotide sequence encoding the

factor VIII<sub>SEP</sub> polypeptide using homologous or site-specific recombination systems (i.e., Cre or FLP recombination systems).

The DNA construct may also contain at least one additional gene to be co-introduced into the host cells.

5           The nucleotide sequences of the present invention can be contained in an expression vector. An "expression vector" is a DNA element, often of circular structure, having the ability to replicate autonomously in a desired host cell, or to integrate into a host cell genome and also possessing certain well-known features which, for example, permit expression of a coding DNA inserted into the vector  
10           sequence at the proper site and in proper orientation. Such features can include, but are not limited to, one or more promoter sequences to direct transcription initiation of the coding DNA and other DNA elements such as enhancers, polyadenylation sites and the like, all as well known in the art.

          Other vectors, including both plasmid and eukaryotic viral vectors, may be  
15           used to express a recombinant gene construct in eukaryotic cells depending on the preference and judgment of the skilled practitioner (see, for example, Sambrook *et al.*, Chapter 16). For example, many viral vectors are known in the art including, for example, retroviruses, adeno-associated viruses, and adenoviruses. Other viruses useful for introduction of a gene into a cell include, but are not limited to, herpes virus,  
20           mumps virus, poliovirus, Sindbis virus, and vaccinia virus, such as, canary pox virus. The methods for producing replication-deficient viral particles and for manipulating the viral genomes are well known. See, for examples, Rosenfeld *et al.* (1991) *Science* 252:431-434, Rosenfeld *et al.* (1992) *Cell* 68:143-155, and U.S. Patent No. 5,882,877 (adenovirus); U.S. Patent No. 5,139,941 (adeno-associated virus); U.S. Patent No.  
25           4,861,719, U.S. Patent No. 5,681,746, and Miller *et al.* (1993) *Methods in Enzymology* 217:581 (retrovirus), all of which are herein incorporated by reference. Therefore, given the knowledge in the art, viral vectors can be readily constructed for use in the introduction of the factor VIII sequences into a cell. Other vectors and expression systems, including bacterial, yeast, and insect cell systems, can be used but  
30           are not preferred due to differences in, or lack of, glycosylation.

          Factor VIII polypeptides of the invention can be expressed in a variety of cells commonly used for culture and recombinant mammalian protein expression. In particular, a number of rodent cell lines have been found to be especially useful hosts



for expression of large proteins. Preferred cell lines, available from the American Type Culture Collection, Rockville, Md., include, but are not limited to, baby hamster kidney cells, and chinese hamster ovary (CHO) cells which are cultured using routine procedures and media. Additional cells of interest can include vertebrate cells such as VERO, HeLa cells, W138, COS-7, and MDCK cell lines. For other suitable expression systems *see* chapters 16 and 17 of Sambrook *et al.* (1989) *Molecular cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, NY). *See*, Goeddel (1990) in *Gene Expression Technology: Methods in Enzymology* 185 (Academic Press, San Diego, CA).

#### Methods of Expression and Isolation

The DNA construct of the present invention may be introduced into a cell (prokaryotic or eukaryotic) by standard methods. As used herein, the terms "transformation" and "transfection" are intended to refer to a variety of art recognized techniques to introduce a DNA into a host cell. Such methods include, for example, transfection, including, but not limited to, liposome-polybrene, DEAE dextran-mediated transfection, electroporation, calcium phosphate precipitation, microinjection, or velocity driven microprojectiles ("biolistics"). Such techniques are well known by one skilled in the art. *See*, Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (2 ed. Cold Spring Harbor Lab Press, Plainview, NY). Alternatively, one could use a system that delivers the DNA construct in a gene delivery vehicle. The gene delivery vehicle may be viral or chemical. Various viral gene delivery vehicles can be used with the present invention. In general, viral vectors are composed of viral particles derived from naturally occurring viruses. The naturally occurring virus has been genetically modified to be replication defective and does not generate additional infectious viruses. The viral vector also contains a DNA construct capable of expressing the factor VIII protein.

The DNA construct containing nucleic acid sequences encoding the factor VIII<sub>SEP</sub> polypeptide may also be administered to cell by a non-viral gene delivery vehicle. Such chemical gene delivery vehicles include, for example, a DNA- or RNA-liposome complex formulation or a naked DNA. *See*, for example, Wang *et al.* (1987) *Proc. Natl. Acad. Sci. U.S.A.* 84:7851, U.S. Patent No. 5,844,107, U.S. Patent

No. 5,108,921, and Wagner *et al.* (1991) *Proc. Natl. Acad. Sci. U.S.A.* 88:4255-4259, all of which are herein incorporated by reference.

It is recognized that the method of introducing the factor VIII<sub>SEP</sub> polypeptide or variant or fragment thereof into a cell can result in either stable integration into the cell genome or transient, episomal expression.

As defined herein, the "expression product" of a DNA encoding a factor VIII<sub>SEP</sub> polypeptide or a fragment or variant thereof is the product obtained from expression of the referenced DNA in a suitable host cell, including such features of pre- or post-translational modification of protein encoded by the referenced DNA, including but not limited to glycosylation, proteolytic cleavage and the like. It is known in the art that such modifications can occur and can differ somewhat depending upon host cell type and other factors, and can result in molecular isoforms of the product, with retention of procoagulant activity. See, for example, Lind *et al.*, (1995) *Eur. J. Biochem.* 232:1927 incorporated herein by reference.

In a one embodiment, cDNA encoding factor VIII<sub>SEP</sub> or a variant or fragment thereof, is inserted in a mammalian expression vector, such as ReNeo. Preliminary characterization of the factor VIII<sub>SEP</sub> is accomplished by transient expression in the ReNeo expression vector containing the factor VIII<sub>SEP</sub> construct in COS-7 cells. A determination of whether active factor VIII<sub>SEP</sub> protein is expressed can then be made. The expression vector construct is used further to stably transfect cells in culture, such as baby hamster kidney cells, using methods that are routine in the art, such as liposome-mediated transfection (Lipofectin.TM., Life Technologies, Inc.). Expression of the factor VIII<sub>SEP</sub> protein can be confirmed, for example, by sequencing, Northern and Western blotting, or polymerase chain reaction (PCR).

Factor VIII<sub>SEP</sub> polypeptides or fragments or variants thereof in the culture media in which the transfected cells stably expressing the protein are maintained can be precipitated, pelleted, washed, and resuspended in an appropriate buffer, and the factor VIII<sub>SEP</sub> protein or variant or fragment thereof is purified by standard techniques, including immunoaffinity chromatography using, for example, monoclonal anti-A2-Sepharose<sup>TM</sup>.

A "fusion protein" or "fusion factor VIII<sub>SEP</sub> or fragment thereof", as used herein, is the product of a hybrid gene in which the coding sequence for one protein is extensively altered, for example, by fusing part of it to the coding sequence for a

second protein from a different gene to produce a hybrid gene that encodes the fusion protein.

In a further embodiment, the factor VIII<sub>SEP</sub> or variant or fragment thereof is expressed as a fusion protein from a recombinant molecule in which sequence  
5 encoding a protein or peptide that enhances, for example, stability, secretion, detection, isolation, or the like is inserted in place adjacent to the factor VIII encoding sequence. See, for example, U.S. Pat. No. 4,965,199 which discloses a recombinant DNA method for producing factor VIII in mammalian host cells and purification of human factor VIII. Human factor VIII expression on CHO (Chinese hamster ovary)  
10 cells and BHKC (baby hamster kidney cells) has been reported. Established protocols for use of homologous or heterologous species expression control sequences including, for example, promoters, operators, and regulators, in the preparation of fusion proteins are known and routinely used in the art. See, Ausubel *et al.* *Current Protocols in Molecular Biology*, Wiley Interscience, N.Y, herein incorporated by  
15 reference. It is further noted that expression is enhanced by including portions of the B-domain. In particular, the inclusion of those parts of the B domain designated "SQ" (Lind *et al.* (1995) *Eur. J. Biochem.* 232:1927, herein incorporated herein by reference) results in favorable expression. "SQ" constructs lack all of the human B domain except for 5 amino acids of the B domain N-terminus and 9 amino acids of  
20 the B domain C-terminus.

It is further recognized that the factor VIII<sub>SEP</sub> polypeptide or variant or fragment thereof of the invention may be prepared via reconstitution methods. In this embodiment factor VIII<sub>SEP</sub>, variants or fragments thereof are made by isolation of subunits, domains, or continuous parts of domains of plasma-derived factor VIII,  
25 followed by reconstitution and purification to produce a factor VIII<sub>SEP</sub> polypeptide of the invention. Alternatively, the factor VIII<sub>SEP</sub>, variant or fragment thereof can be made by recombinant DNA methods, followed by reconstitution and purification.

More particularly, the method of preparing a factor VIII<sub>SEP</sub> by reconstitution methods can be performed via a modification of procedures reported by Fay *et al.*  
30 (1990) *J. Biol. Chem.* 265:6197; and Lollar *et al.* (1988) *J. Biol. Chem.* 263:10451, which involves the isolation of subunits (heavy and light chains) of human and animal factor VIII, followed by recombination of human heavy chain and animal light chain or by recombination of human light chain and animal heavy chain.

Isolation of both human and animal individual subunits involves dissociation of the light chain/heavy chain dimer. This is accomplished, for example, by chelation of calcium with ethylenediaminetetraacetic acid (EDTA), followed by monoS<sup>TM</sup> HPLC (Pharmacia-LKB, Piscataway, N.J.). Hybrid human/animal factor VIII molecules are reconstituted from isolated subunits in the presence of calcium. Hybrid human light chain/animal heavy chain or animal light chain/human heavy chain factor VIII is isolated from unreacted heavy chains by monoS<sup>TM</sup> HPLC by procedures for the isolation of porcine factor VIII, such as described by Lollar *et al.* (1988) *Blood* 71:137-143 and in U.S. Patent No. 6,376,463, both of which is herein incorporated by reference.

### Diagnostic Assays

As used herein, "diagnostic assays" include assays that in some manner utilize the antigen-antibody interaction to detect and/or quantify the amount of a particular antibody that is present in a test sample to assist in the selection of medical therapies. There are many such assays known to those of skill in the art. As used herein, however, the factor VIII<sub>SEP</sub> DNA or variant or fragment thereof and protein expressed therefrom, in whole or in part, can be substituted for the corresponding reagents in the otherwise known assays, whereby the modified assays may be used to detect and/or quantify antibodies to factor VIII. It is the use of these reagents, the factor VIII<sub>SEP</sub> DNA or variants or fragments thereof or protein expressed therefrom, that permits modification of known assays for detection of antibodies to human or animal factor VIII or to hybrid human/animal factor VIII. As used herein, the factor VIII<sub>SEP</sub> or variants or fragment thereof that includes at least one epitope of the protein can be used as the diagnostic reagent.

The DNA or amino acid sequence of the factor VIII<sub>SEP</sub> or variant or fragment thereof can be used in assays as diagnostic reagents for the detection of inhibitory antibodies to human or animal factor VIII, including, for example, samples of serum and body fluids of human patients with factor VIII deficiency. These antibody assays include assays such as ELISA assays, immunoblots, radioimmunoassays, immunodiffusion assays, and assay of factor VIII biological activity (e.g., by coagulation assay). Examples of other assays in which the factor VIII<sub>SEP</sub> or variant or fragment thereof can be used include the Bethesda assay and anticoagulation assays.

Techniques for preparing these reagents and methods for use thereof are known to those skilled in the art. For example, an immunoassay for detection of inhibitory antibodies in a patient serum sample can include reacting the test sample with a sufficient amount of the factor VIII<sub>SEP</sub> that contains at least one antigenic site,  
5 wherein the amount is sufficient to form a detectable complex with the inhibitory antibodies in the sample.

Nucleic acid and amino acid probes can be prepared based on the sequence of the factor VIII<sub>SEP</sub> DNA or protein molecule or fragments or variants thereof. In some embodiments, these can be labeled using dyes or enzymatic, fluorescent,  
10 chemiluminescent, or radioactive labels that are commercially available. The amino acid probes can be used, for example, to screen sera or other body fluids where the presence of inhibitors to human, animal, or hybrid human/animal factor VIII is suspected. Levels of inhibitors can be quantitated in patients and compared to healthy controls, and can be used, for example, to determine whether a patient with a factor  
15 VIII deficiency can be treated with a factor VIII<sub>SEP</sub> or active fragment or variant thereof. The cDNA probes can be used, for example, for research purposes in screening DNA libraries.

#### Pharmaceutical Compositions

20 The present invention further provides pharmaceutical compositions comprising the nucleic acid molecules and the polypeptides encoding the high-level expression factor VIII<sub>SEP</sub> of the present invention or variants and fragments thereof. Such compositions can comprise nucleic acids and polypeptides of the invention either alone or in combination with appropriate pharmaceutical stabilization  
25 compounds, delivery vehicles, and/or carrier vehicles, are prepared according to known methods, as described in Martin *et al. Remington's Pharmaceutical Sciences*, herein incorporated by reference.

In one embodiment, the preferred carriers or delivery vehicles for intravenous infusion are physiological saline or phosphate buffered saline.

30 In another embodiment, suitable stabilization compounds, delivery vehicles, and carrier vehicles include but are not limited to other human or animal proteins such as albumin.

Phospholipid vesicles or liposomal suspensions may also be used as pharmaceutically acceptable carriers or delivery vehicles. These can be prepared according to methods known to those skilled in the art and can contain, for example, phosphatidylserine-phosphatidylcholine or other compositions of phospholipids or  
5 detergents that together impart a negative charge to the surface, since factor VIII binds to negatively charged phospholipid membranes. Liposomes may be prepared by dissolving appropriate lipid(s) (such as stearyl phosphatidyl ethanolamine, stearyl phosphatidyl choline, arachadoyl phosphatidyl choline, and cholesterol) in an inorganic solvent that is then evaporated, leaving behind a thin film of dried lipid on  
10 the surface of the container. An aqueous solution of the factor VIII<sub>SEP</sub> of the present invention is then introduced into the container. The container is then swirled by hand to free lipid material from the sides of the container and to disperse lipid aggregates, thereby forming the liposomal suspension.

The factor VIII<sub>SEP</sub> molecules of the invention can be combined with other  
15 suitable stabilization compounds, delivery vehicles, and/or carrier vehicles, including vitamin K dependent clotting factors, tissue factor, and von Willebrand factor (vWf) or a fragment of vWf that contains the factor VIII binding site, and polysaccharides such as sucrose.

Factor VIII<sub>SEP</sub> molecules of the invention can also be delivered by gene  
20 therapy using delivery means such as retroviral vectors. This method consists of incorporation of a nucleotide sequence encoding desired factor VIII<sub>SEP</sub> polypeptide of the invention into human cells that are transplanted directly into a factor VIII<sub>SEP</sub> deficient patient or that are placed in an implantable device, permeable to the factor VIII molecules but impermeable to cells, that is then transplanted.

25 In one embodiment, the method will be retroviral-mediated gene transfer. In this method, a nucleotide sequence encoding a factor VIII polypeptide of the invention is cloned into the genome of a modified retrovirus. The gene is inserted into the genome of the host cell by viral machinery where it will be expressed by the cell. The retroviral vector is modified so that it will not produce virus, preventing  
30 viral infection of the host. The general principles for this type of therapy are known to those skilled in the art and have been reviewed in the literature (Kohn *et al.* (1989) *Transfusion* 29:812-820).

The factor VIII<sub>SEP</sub> polypeptide of the invention can be stored bound to vWf to increase the half-life and shelf-life of the polypeptide molecule. Additionally, lyophilization of factor VIII<sub>SEP</sub> can improve the yields of active molecules in the presence of vWf. Current methods for storage of human and animal factor VIII used by commercial suppliers can be employed for storage of recombinant factor VIII. These methods include: (1) lyophilization of factor VIII<sub>SEP</sub> in a partially-purified state (as a factor VIII "concentrate" that is infused without further purification); (2) immunoaffinity-purification of factor VIII<sub>SEP</sub> by the Zimmerman method and lyophilization in the presence of albumin, which stabilizes the factor VIII; (3) lyophilization of recombinant factor VIII<sub>SEP</sub> in the presence of albumin.

Additionally, the factor VIII polypeptides can be stored at 4° C in 0.6 M NaCl, 20 mM MES, and 5 mM CaCl<sub>2</sub> at pH 6.0. The polypeptides can also be stored frozen in these buffers and thawed with minimal loss of activity.

#### 15 Methods of Treatment

Factor VIII<sub>SEP</sub> or fragments and variant thereof can be used to treat uncontrolled bleeding due to factor VIII deficiency (e.g., intraarticular, intracranial, or gastrointestinal hemorrhage) in hemophiliacs with and without inhibitory antibodies and in patients with acquired factor VIII deficiency due to the development of inhibitory antibodies. The active materials are preferably administered intravenously.

"Factor VIII deficiency," as used herein, includes deficiency in clotting activity caused by production of defective factor VIII, by inadequate or no production of factor VIII, or by partial or total inhibition of factor VIII by inhibitors. Hemophilia A is a type of factor VIII deficiency resulting from a defect in an X-linked gene and the absence or deficiency of the factor VIII protein it encodes.

Additionally, factor VIII<sub>SEP</sub> or fragments and variant thereof can be administered by transplantation of cells genetically engineered to produce the factor VIII<sub>SEP</sub> or by implantation of a device containing such cells, as described above.

In one embodiment, pharmaceutical compositions of factor VIII<sub>SEP</sub> or fragments and variants thereof alone or in combination with stabilizers, delivery vehicles, and/or carriers are infused into patients intravenously according to the same procedure that is used for infusion of factor VIII<sub>SEP</sub>.

The treatment dosages of the factor VIII<sub>SEP</sub> composition or variants or fragments thereof that must be administered to a patient in need of such treatment will vary depending on the severity of the factor VIII deficiency. Generally, dosage level is adjusted in frequency, duration, and units in keeping with the severity and duration of each patient's bleeding episode. Accordingly, the factor VIII<sub>SEP</sub> or variants or fragments thereof is included in the pharmaceutically acceptable carrier, delivery vehicle, or stabilizer in an amount sufficient to deliver to a patient a therapeutically effective amount of the hybrid to stop bleeding, as measured by standard clotting assays.

"Specific activity" as used herein, refers to the activity that will correct the coagulation defect of human factor VIII deficient plasma. Specific activity is measured in units of clotting activity per milligram total factor VIII protein in a standard assay in which the clotting time of human factor VIII deficient plasma is compared to that of normal human plasma. One unit of factor VIII activity is the activity present in one milliliter of normal human plasma. In the assay, the shorter the time for clot formation, the greater the activity of the factor VIII being assayed. The specific activity of the factor VIII polypeptides, variant or fragments thereof, may be less than, equal to, or greater than that of either plasma-derived or recombinant human factor VIII.

Factor VIII is classically defined as that substance present in normal blood plasma that corrects the clotting defect in plasma derived from individuals with hemophilia A. The coagulant activity *in vitro* of purified and partially-purified forms of factor VIII<sub>SEP</sub> is used to calculate the dose of factor VIII for infusions in human patients and is a reliable indicator of activity recovered from patient plasma and of correction of the *in vivo* bleeding defect. There are no reported discrepancies between standard assay of novel factor VIII molecules *in vitro* and their behavior in the dog infusion model or in human patients, according to Lusher *et al.* *New Engl. J. Med.* 328:453-459; Pittman *et al.* (1992) *Blood* 79:389-397; and Brinkhous *et al.* (1985) *Proc. Natl. Acad. Sci.* 82:8752-8755.

The increase of factor VIII<sub>SEP</sub> in the plasma will be sufficient to produce a therapeutic effect. A "therapeutic effect" is defined as an increase in the blood coagulation activity in the plasma of patients that is greater than the coagulation activity observed in the subject before administration of the factor VIII<sub>SEP</sub> molecule.



In a standard blood clotting assay, the shorter time for clot formation, the greater the activity of factor VIII being assayed. An increase in factor VIII activity in the factor VIII deficient plasma of at least 1% or higher will be therapeutically beneficial.

Usually, the desired plasma factor VIII level to be achieved in the patient  
5 through administration of the factor VIII<sub>SEP</sub> or variant or fragment thereof is in the range of 30-100% of normal. In a one mode of administration of the factor VIII<sub>SEP</sub> or fragment or variant thereof, the composition is given intravenously at a preferred dosage in the range from about 5 to 50 units/kg body weight, more preferably in a range of 10-50 units/kg body weight, and most preferably at a dosage of 20-40  
10 units/kg body weight; the interval frequency is in the range from about 8 to 24 hours (in severely affected hemophiliacs); and the duration of treatment in days is in the range from 1 to 10 days or until the bleeding episode is resolved. See, for example, Roberts *et al.* (1990) *Hematology*, Williams *et al.* ed. Ch. 153, 1453-1474, herein incorporated by reference. Patients with inhibitors may require more factor VIII<sub>SEP</sub> or  
15 variants or fragments thereof, or patients may require less factor VIII<sub>SEP</sub> or fragments or variants thereof. As in treatment with human or porcine factor VIII, the amount of factor VIII<sub>SEP</sub> or fragments or variants infused is defamed by the one-stage factor VIII coagulation assay and, in selected instances, *in vivo* recovery is determined by measuring the factor VIII in the patient's plasma after infusion. It is to be understood  
20 that for any particular subject, specific dosage regimens should be adjusted over time according to the individual need and the professional judgment of the person administering or supervising the administration of the compositions, and that the concentration ranges set forth herein are exemplary only and are not intended to limit the scope or practice of the claimed composition.

25 Treatment can take the form of a single intravenous administration of the composition or periodic or continuous administration over an extended period of time, as required. Alternatively, factor VIII<sub>SEP</sub> or fragments or variants thereof can be administered subcutaneously or orally with liposomes in one or several doses at varying intervals of time.

30 Factor VIII<sub>SEP</sub> or fragments or variants thereof can also be used to treat uncontrolled bleeding due to factor VIII deficiency in hemophiliacs who have developed antibodies to human factor VIII.

## EXPERIMENTAL

### EXAMPLE 1

#### Sequence Characterization of Factor VIII

Both porcine and human factor VIII are isolated from plasma as a two subunit  
5 protein. The subunits, known as the heavy chain and light chain, are held together by  
a non-covalent bond that requires calcium or other divalent metal ions. The heavy  
chain of factor VIII contains three domains, A1, A2, and B, which are linked  
covalently. The light chain of factor VIII also contains three domains, designated A3,  
C1, and C2. The B domain has no known biological function and can be removed, or  
10 partially removed from the molecule proteolytically or by recombinant DNA  
technology methods without significant alteration in any measurable parameter of  
factor VIII. Human recombinant factor VIII has a similar structure and function to  
plasma-derived factor VIII, though it is not glycosylated unless expressed in  
mammalian cells. Both human and porcine activated factor VIII ("factor VIIIa") have  
15 three subunits due to cleavage of the heavy chain between the A1 and A2 domains.  
This structure is designated A1/A2/A3-C1-C2.

The cDNA sequence of porcine factor VIII corresponding the signal peptide  
coding region, the A1, B, light chain activity peptide region A3, C1, and C2 domains  
is provided in SEQ ID NO:1. The translation of the porcine cDNA is provided in  
20 SEQ ID NO:2.

The alignment of the predicted amino acid sequence of full-length porcine  
factor VIII (SEQ ID NO:2) with the published human (Wood *et al.* (1984) *Nature*  
312:330-337) (SEQ ID NO:6) and murine (Elder *et al.* (1993) *supra*) (SEQ ID NO:8)  
sequences are shown in Figures 1A-1H along with sites for post-translational  
25 modification, proteolytic cleavage, and recognition by other macromolecules.

Potential N-linked glycosylation sites (NXS/T where X is not proline) can be  
seen in Figures 1A-1H. There are eight conserved N-linked glycosylation sites: one  
in the A1 domain, one in the A2 domain, four in the B domain, one in the A3 domain,  
and one in the C1 domain. The 19 A and C domain cysteines are conserved, whereas  
30 there is divergence of B domain cysteines. Six of the seven disulfide linkages in  
factor VIII are found at homologous sites in factor V and Ceruloplasmin, and both C  
domain disulfide linkages are found in factor V (McMullen *et al.* (1995) *Protein Sci.*  
4:740-746). Human factor VIII contains sulfated tyrosines at positions 346, 718, 719,

723, 1664, and 1680 (Pittman *et al.* (1992) *Biochemistry* 31:3315-3325; Michnick *et al.* (1994) *J. Biol. Chem.* 269:20095-20102). These residues are conserved in mouse factor VIII and porcine factor VIII (Figure 1), although the CLUSTALW program failed to align the mouse tyrosine corresponding to Tyr346 in human factor VIII.

5 Epitopes of the various domain of the factor VIII polypeptide are outlined in Figure 1.

## EXAMPLE 2

### Summary

Human factor VIII expression levels are significantly lower than levels of  
10 other coagulation proteins *in vivo* and in heterologous expression systems *in vitro*. Low-level expression of recombinant human factor VIII has constrained the treatment of hemophilia A using recombinant protein infusion and gene therapy protocols. However, recombinant B-domain-deleted porcine factor VIII is expressed at levels 10–14 fold greater than recombinant B-domain-deleted human factor VIII *in vitro*.  
15 To identify sequences of porcine factor VIII necessary for this property, B-domain-deleted human/porcine hybrid factor VIII cDNAs were produced that contained substitution of human sequences with the corresponding porcine sequences. These cDNAs were transiently transfected into COS-7 cells or stably transfected into BHK-derived cells and factor VIII expression into the extracellular media was measured by  
20 one-stage coagulation assay. Human/porcine hybrid factor VIII cDNAs containing 1) the A1, A2 and A3 domains of porcine factor VIII and the C1 and C2 domains of human factor VIII, or 2) the A1 and A3 domains of porcine factor VIII and the A2, C1, and C2 domains of human factor VIII demonstrated factor VIII expression levels comparable to porcine factor VIII. A human/porcine hybrid factor VIII molecule  
25 demonstrating high-level expression may be valuable for improving factor VIII production for intravenous infusion or for somatic cell gene therapy of hemophilia A.

### Materials

Dulbecco's phosphate-buffered saline, fetal bovine serum (FBS), penicillin,  
30 streptomycin, DMEM:F12, serum-free AIM V culture media, Lipofectin, Lipofectamine 2000 and geneticin were purchased from Invitrogen. Baby hamster kidney - derived cells, designated BHK-M cells (Funk *et al.* (1990) *Biochemistry* 29:1654-1660), were a gift from Dr. Ross Macgillivray, University of British Columbia. Transient transfections were controlled for transfection efficiency using RTA01/2152497v1

the RL-CMV vector and Dual-Luciferase Assay Kit (Promega, Madison, WI). Citrated factor VIII-deficient plasma and pooled citrated normal human plasma (FACT) were purchased from George King Biomedical (Overland Park, KA). Activated partial thromboplastin reagent (aPTT) was purchased from Organon  
5 Teknika (Durham, NC). Oligonucleotide primers were synthesized by Life Technologies. *Pfu* DNA polymerase and *E. coli* XL-1 Blue cells were purchased from Stratagene (La Jolla, CA).

#### Construction of Factor VIII expression vectors

10 All of the factor VIII expression vectors in this study were contained in the ReNeo mammalian expression plasmid (Lind *et al.* (1995) *Eur.J.Biochem.* 232:19-27). The factor VIII cDNA inserts lack endogenous factor VIII 5'-UTR sequence and contain the first 749 of the 1805 nt human factor VIII 3'-UTR.

A human B domain-deleted factor VIII cDNA designed HSQ (Figure 2) was  
15 created by cloning the human factor VIII cDNA into the mammalian expression vector ReNeo as described previously (Doering *et al.* (2002) *J.Biol.Chem.* 277: 38345-38349). The HSQ cDNA encodes an S F S Q N P P V L K R H Q R (SEQ ID NO:9) linker sequence between the A2 and *ap* domains. This linker includes the R H Q R (SEQ ID NO:10) recognition sequence for intracellular proteolytic processing by  
20 PACE/furin (Seidah *et al.* (1997) *Current Opinion in Biotechnology* 8:602-607). This cleavage event converts single chain factor VIII into a heterodimer (Lind *et al.* (1995) *Eur.J.Biochem.* 232:19-27). Heterodimeric factor VIII is considered the physiologic form of factor VIII (Fass *et al.* (1982) *Blood* 59:594-600).

A B-domain-deleted form of porcine factor VIII cDNA was ligated into  
25 ReNeo as described previously (Doering *et al.* (2002) *J.Biol.Chem.* 277: 38345-38349). The cDNA, designated P/OL (Figure 2), encodes a porcine-derived linker sequence S F A Q N S R P P S A S A P K P P V L R R H Q R (SEQ ID NO:11) between the A2 and *ap* domains for PACE/furin recognition.

A B-domainless hybrid human/porcine factor VIII molecule designated HP1,  
30 which contains the porcine A2 domain and human A1, *ap*-A3, C1 and C2 domains, was prepared as described previously (Lubin *et al.* (1994) *J.Biol.Chem.* 269:8639-8641). The cDNA encoding the human-derived linker sequence S F S Q N P P V L K R H Q R (SEQ ID NO:9) was inserted between the A2 and *ap* domains of HP1 by

splicing-by-overlap extension (SOE) mutagenesis (Horton *et al.* (1993) *Methods Enzymol.* 217:270-279), producing HP1/SQ (Figure 2).

HP30, which contains the porcine *ap*-A3 domain and human A1, A2, C1 and C2 domains, was prepared as described previously (Barrow *et al.* (2000) *Blood* 95:557-561). The cDNA encoding the porcine-derived linker sequence S F A Q N S R P P S A S A P K P P V L R R H Q R (SEQ ID NO:11) was inserted between the A2 and *ap* domains of HP30 by SOE mutagenesis, producing HP30/OL (Figure 2).

HP44/OL, which contains the porcine A1, A2, *ap*-A3 domains, the porcine-derived linker sequence S F A Q N S R P P S A S A P K P P V L R R H Q R (SEQ ID NO:11) and the human C1 and C2 domains (Figure 2), was prepared as follows. P/OL ReNeo was digested with *AvrII* and the fragment containing A1, A2 and ReNeo sequence was gel purified. HP30/OL was digested with *AvrII* and the fragment containing porcine *ap*-A3 and human C1 and C2 sequences was gel purified. Ligation of the products, transformation of *E. coli* XL-1 cells and plasmid purification were performed as described previously (Healey *et al.* (1998) *Blood* 92:3701-3709).

HP46/SQ, which contains the porcine A1 domain and human A2, *ap*-A3, C1 and C2 domains and the human S F S Q N P P V L K R H Q R (SEQ ID NO:11) linker sequence (Figure 2), was prepared by SOE mutagenesis. P/OL in ReNeo and HSQ in ReNeo were used as templates in the first round SOE reactions. The 5' primer in the P/OL reaction was complementary to ReNeo sequence 5' to the factor VIII cDNA. The 3' primer flanked the porcine A1 domain. The 5' primer in the HSQ reaction was partially complementary to the 3' primer used in the first reaction. The 3' primer was complementary to human A2 sequence. Following gel purification of the products from the first round reactions, the second SOE reaction was performed, yielding a product containing ReNeo sequence 5' to the factor VIII cDNA insert, the porcine A1 domain, and part of the human A2 domain. This product was digested with *XhoI*, at the junction of ReNeo and the factor VIII insert, and *MluI*, in the human A2 domain, and ligated into *XhoI/MluI* digested HSQ/ReNeo. The resulting plasmid was amplified by transformation into *E. coli* XL-1 Blue cells as described above.

HP47/OL, which contains the porcine A1, *ap*-A3 domains, porcine-derived linker sequence S F A Q N S R P P S A S A P K P P V L R R H Q R (SEQ ID NO:11) and human A2, C1 and C2 domains (Figure 2) was prepared as follows. HP46/SQ in ReNeo was digested with *AvrII*, which cleaves the plasmid in the ReNeo

sequence 5 to the factor VIII insert and at the A2-*ap* junction. The fragment containing the A1 and A2 domains was gel purified ligated to a fragment of HP30/OL in ReNeo produced by *AvrII* digestion.

Sequences produced by SOE mutagenesis were confirmed by dideoxy DNA sequencing.

#### Transient expression of Factor VIII from COS-7 cells

COS-7 cells were grown to 70 – 80% confluence in 2 cm<sup>2</sup> wells containing 1 ml DMEM:F12 supplemented with 10% FBS, 100 units/ml penicillin and 100 µg/ml streptomycin. Cells were transfected with a 2000:1 mass ratio of factor VIII plasmid:luciferase plasmid DNA using Lipofectamine 2000. Twenty-four hours after transfection the cells were rinsed twice with 1 ml of PBS and 0.5 ml of serum-free AIM V medium was added to each well. Cells were cultured 24 hr before the conditioned media was harvested and factor VIII activity was measured as described below.

#### Stable expression of Factor VIII from baby hamster kidney-derived (BHK-M) cells

BHK-M cells were transfected using Lipofectin along with an ReNeo plasmid containing factor VIII cDNA and cultured in the presence of DMEM:F12 containing 10% FBS, 100 units/ml penicillin, 100 µg/ml streptomycin and 500 µg/ml geneticin for 10 days. The ReNeo vector contains the neomycin phosphotransferase gene for resistance to the antibiotic geneticin. Twenty-four to 72 geneticin resistant clones were screened for factor VIII production. The clone from each cDNA construct that displayed the highest level of factor VIII activity was transferred into a 75 cm<sup>2</sup> flask, grown to 90 – 95% confluence and then switched to 25 ml serum-free AIM V media. After 24 hr, the conditioned media was replaced with 25 ml fresh serum-free media AIM V and cultured for an additional 24 hr. Harvested media from each time point was assayed for factor VIII activity as described below.

#### Factor VIII assay

Factor VIII activity was measured by one-stage coagulation assay using a ST art Coagulation Instrument (Diagnostica Stago, Asnieres, France). Five µl of sample or standard was added to 50 µl of factor VIII-deficient plasma, followed by addition

of 50 µl aPTT reagent and incubation for 3 min at 37°C. Fifty microliters of 20 mM CaCl<sub>2</sub> was added to initiate the reaction, and the time required to develop a fibrin clot was measured viscometrically. Standard curves were generated using several dilutions of pooled normal human plasma and subjected to linear regression analysis of the clotting time versus the logarithm of the reciprocal plasma dilution. For determination of factor VIII activity, samples were diluted in HEPES buffered saline to a concentration within the range of the standard curve.

## RESULTS

To identify regions in porcine factor VIII that confer high-level expression, human/porcine hybrid factor VIII molecules shown in Figure 2 were constructed and their expression levels in COS-7 and BHK-M cells were measured. After COS-7 cell transfection, the expression plasmid is not integrated into genomic DNA, but is present transiently as an episomal DNA. Expression levels from COS-7 cells represent an average of the cell population. Figure 3 shows the results of COS-7 wells transfected in quadruplicate. There is a significant increase in expression of P/OL, HP44, HP47, and HP46 compared to HSQ. In contrast, expression of HP1 and HP30 were not increased compared to HSQ.

Expression of factor VIII from BHK-M cells was consistent with the results in COS-7 cells. After BHK-M cell transfection, clones containing plasmid DNA that is stably incorporated into the genome are selected using the antibiotic geneticin. Cells that do not contain the neomycin phosphotransferase gene contained in the plasmid do not survive in the presence of geneticin. Approximately 50% of the clones resulting from transfection of BHK-M cells with the constructs shown in Figure 2 did not express detectable levels of factor VIII (data not shown). This is consistent with previous results with HSQ and P/OL (Doering *et al.* (2002) *J.Biol.Chem.* 277: 38345-38349) and is expected because factor VIII expression *per se* is not selected for during geneticin selection. Average expression levels for factor VIII-producing clones were significantly higher for the P/OL, HP44, HP47, and HP46, but not the HP1 and HP30 constructs, compared to HSQ (data not shown). For each factor VIII cDNA construct, the clone producing the highest levels of factor VIII was expanded and switched to serum-free AIM V medium. Consistent with the above results, factor VIII levels for

the HP44, HP47, and HP46, but not the HP1 and HP30, were comparable to P/OL (Figure 3).

Figure 3 shows heterologous expression of recombinant porcine factor VIII OL and recombinant human factor VIII SQ. COS-7 cells (solid bars) were transfected with the individual factor VIII expression constructs and luciferase plasmid DNA and cultured in serum-free media for 24 hr as described in Experimental Procedures. Conditioned media was assayed for factor VIII activity by one-stage coagulation assay. After media harvest, cells were lysed and assayed for luciferase activity. Data are presented as the ratio of factor VIII activity:luciferase activity (mean +/- standard deviation of four wells of transfected cells for each sample) normalized to the mean HSQ level. Data shown are representative of experiments involving three separate cultures of COS-7 cells. BHK-M cells (hatched bars) were transfected with the individual factor VIII expression constructs and selected for stable transgene integration. The top producing clone for each construct was split to a 75 cm<sup>2</sup> flask, grown to greater than 90% confluence, rinsed twice with PBS and cultured 24 hr in serum-free media. After 24 hr, the media was harvested and assayed for factor VIII activity. The data are expressed relative to HSQ expression, which was 2.8 units/10<sup>6</sup> cells/24 h in BHK-M cells.

## DISCUSSION

Recombinant B domain-deleted porcine factor VIII is expressed at levels up to 14-fold greater than recombinant human factor VIII (Doering *et al.* (2002) *J.Biol.Chem.* 277: 38345-38349). The levels are substantially greater than in previously published reports of factor VIII expression (Table II). The mechanism for the high expression phenomenon has not been established. However, high-level expression is due to a difference between human and porcine B domain-deleted factor VIII in translated sequence because the P/OL and HSQ expression cassettes do not contain endogenous factor VIII 5'- UTR sequence, while both possess the first 749 nt (of 1805 nt) of the human factor VIII 3'UTR. Furthermore, the effect occurs at the post-transcriptional level, because there is no difference in P/OL and HSQ mRNA levels in BHK-M cells (Doering *et al.* (2002) *J.Biol.Chem.* 277: 38345-38349).



TABLE II. Previous Reports of FACTOR VIII Expression.

| FACTOR VIII Construct     | FVIII Level                            | Assay                  | Serum | vWf | Cell Line        | Reference  |
|---------------------------|--|------------------------|-------|-----|------------------|--|
| Human, full length        | 0.07 <sup>a</sup>                      | Coatest                | +     | -   | BHK              | Wood <i>et al.</i> (1984) <i>Nature</i> 312:330-337                          |
| Human, full length        | 0.16 <sup>a</sup><br>0.33 <sup>a</sup> | Coatest<br>Coagulation | +     | -   | COS              | Toole <i>et al.</i> (1986) <i>Proc. Natl. Acad. Sci. U.S.A.</i> 83:5939-5942 |
| Human, B domain-deleted   | 0.34 <sup>a</sup>                      | Coatest                | -     | -   | CHO <sup>c</sup> | Kaufman <i>et al.</i> (1988) <i>J. Biol. Chem.</i> 263:6352-6362             |
| Human, full length        | 1.4 <sup>b</sup>                       | Coatest                | -     | +   | CHO              | Kaufman <i>et al.</i> (1989) <i>Mol. Cell Biol.</i> 9:1233-1242              |
| Human, B domain-deleted   | 5 <sup>a</sup>                         | Coatest                | -     | +   | CHO              | Pittman <i>et al.</i> (1993) <i>Blood</i> 81:2925-2935                       |
| Human, B domain-deleted   | 1.5 <sup>a</sup>                       | Coatest                | -     | -   | CHO              | Lind <i>et al.</i> (1995) <i>Eur. J. Biochem.</i> 232:19-27                  |
| Human, B domain-deleted   | 2.5 <sup>b</sup>                       | Coagulation            | +     | -   | CHO              | Plantier <i>et al.</i> (2001) <i>Thromb. Haemost.</i> 86:596-603             |
| Human, B domain-deleted   | 3.1 <sup>a</sup><br>10 <sup>b</sup>    | Coagulation            | -     | -   | BHK              | Doering <i>et al.</i> (2002) <i>J. Biol. Chem.</i> 277, 38345-38349          |
| Porcine, B domain-deleted | 41 <sup>a</sup><br>140 <sup>b</sup>    | Coagulation            | -     | -   | BHK              | Doering <i>et al.</i> (2002) <i>J. Biol. Chem.</i> 277, 38345-38349          |

<sup>a</sup> units/milliliter/24 hours<sup>b</sup> units/10<sup>6</sup> cells/24 hours<sup>c</sup> Chinese hamster ovary

## EXAMPLE 3

Variants of the factor VIII<sub>SEP</sub> sequences of the invention may be generated.

For example, the HP63/OL factor VIII<sub>SEP</sub> may be generated. See Figures 12-14.

- Two major human factor VIII epitopes that are recognized by inhibitory antibodies have been identified: in the A2 domain in a segment bound by residues 484 - 508 (Healey *et al.* (1995) *J. Biol. Chem.* 270:14505-14509) and in the C2 domain in a segment bounded by residues 2181 - 2252 (Healey *et al.* (1998) *Blood* 92:3701-3709 and Barrow *et al.* (2001) *Blood* 97:169-174, all of which are herein incorporated by

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reference). The sequence numbering refers to the full-length, mature human factor VIII according to standard convention (Vehar *et al.* (1984) *Nature* 312:337-342). Antibodies also have been identified that recognize the light chain activation peptide, *ap*, (Barrow *et al.* (2000) *Blood* 95:557-561) and the A3 domain in a region bounded  
5 by residues 1804 - 1819 (Zhong *et al.* (1998) *Blood* 92:136-142), but they are less common (Prescott *et al.* (1997) *Blood* 89:3663-3671). Other epitopes occasionally have been identified, but they are considered unusual.

A variant of a factor VIII<sub>SEP</sub> molecule can be generated to contain the human A2, *ap*, and C2 domains, human sequence 1804-1819 and the porcine A1 domain and  
10 porcine A3 sequences from about 1690 to 1803 and from about 1820 to 2019. This factor VIII<sub>SEP</sub> variant is diagramed in Figure 12 as HP63. The amino acid and nucleotide sequences are provided in SEQ ID NO: 20 and 21. Such a molecule is predicted to be a super-expresser that has the antigenic characteristics of human factor VIII. Assays to measure the high-level expression activity of the HP63 variant are  
15 disclosed elsewhere herein.

Table III Sequence ID Listing

| SEQ ID NO | Type | Species             | Description   |
|-----------|------|---------------------|---|
| 1         | NT   | <i>Sus scrofa</i>   | Factor VIII   |
| 2         | AA   | <i>Sus scrofa</i>   | Factor VIII   |
| 3         | NT   | <i>Sus scrofa</i>   | Factor VIII – B-domain deleted (retains first 12 and last 12 amino acids of B-domain)   |
| 4         | AA   | <i>Sus scrofa</i>   | Factor VIII – B-domain deleted (retains first 12 and last 12 amino acids of B-domain)   |
| 5         | NT   | <i>Homo sapiens</i> | Factor VIII with 5' and 3' UTR sequences  |
| 6         | AA   | <i>Homo sapiens</i> | Factor VIII   |
| 7         | NT   | <i>Homo sapiens</i> | Factor VIII cDNA  |
| 8         | AA   | <i>Mus musculus</i> | Factor VIII   |
| 9         | AA   | <i>Homo sapiens</i> | Linker sequence between A2 and <i>ap</i> domains  |
| 10        | AA   | <i>Homo sapiens</i> | Recognition sequence for PACE/furin   |
| 11        | AA   | <i>Sus scrofa</i>   | Linker sequence between A2 and <i>ap</i> domains  |
| 12        | NT   | <i>Homo sapiens</i> | Factor VIII – B-domain deleted  |
| 13        | AA   | <i>Homo sapiens</i> | Factor VIII – B domain deleted  |
| 14        | NT   | Artificial          | HP44/OL Factor VIII which has the following domains: A1 <sub>p</sub> -A2 <sub>p</sub> - <i>ap</i> <sub>p</sub> -A3 <sub>p</sub> -C1 <sub>H</sub> -C2 <sub>H</sub> |
| 15        | AA   | Artificial          | HP44/OL Factor VIII which has the following domains: A1 <sub>p</sub> -A2 <sub>p</sub> - <i>ap</i> <sub>p</sub> -A3 <sub>p</sub> -C1 <sub>H</sub> -C2 <sub>H</sub> |
| 16        | NT   | Artificial          | HP46/SQ Factor VIII which has the following domains: A1 <sub>p</sub> -A2 <sub>H</sub> - <i>ap</i> <sub>H</sub> -A3 <sub>H</sub> -C1 <sub>H</sub> -C2 <sub>H</sub> |
| 17        | AA   | Artificial          | HP46/SQ Factor VIII which has the following domains: A1 <sub>p</sub> -A2 <sub>H</sub> - <i>ap</i> <sub>H</sub> -A3 <sub>H</sub> -C1 <sub>H</sub> -C2 <sub>H</sub> |
| 18        | NT   | Artificial          | HP47/OL Factor VIII which has the following domains: A1 <sub>p</sub> -A2 <sub>H</sub> - <i>ap</i> <sub>p</sub> -A3 <sub>p</sub> -C1 <sub>H</sub> -C2 <sub>H</sub> |
| 19        | AA   | Artificial          | HP47/OL Factor VIII which has the following domains: A1 <sub>p</sub> -A2 <sub>H</sub> - <i>ap</i> <sub>p</sub> -A3 <sub>p</sub> -C1 <sub>H</sub> -C2 <sub>H</sub> |
| 20        | NT   | Artificial          | HP63/OL   |
| 21        | AA   | Artificial          | HP63/OL   |

The present invention has been described above with reference to the accompanying drawings, in which some, but not all embodiments of the invention are shown. Indeed, these inventions may be embodied in many different forms and should not be construed as limited to the embodiments set forth herein; rather, these embodiments are provided so that this disclosure will satisfy applicable legal requirements. Like numbers refer to like elements throughout.

Many modifications and other embodiments of the inventions set forth herein will come to mind to one skilled in the art to which these inventions pertain having the benefit of the teachings presented in the foregoing descriptions and the associated drawings. Therefore, it is to be understood that the inventions are not to be limited to

the specific embodiments disclosed and that modifications and other embodiments are intended to be included within the scope of the appended claims. Although specific terms are employed herein, they are used in a generic and descriptive sense only and not for purposes of limitation.

5           All publications and patent applications mentioned in the specification are indicative of the level of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

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